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plastids undifferentiated, a nuclear mutation that disrupts plastid differentiation in Zea mays L.

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Abstract Photosynthetic development in any plant requires the intracellular co-ordination of chloroplast and nuclear gene expression programs. In this report, we investigate the role of a nuclear gene in photosynthetic development by examining C4 photosynthetic differentiation in a yellow mutant of maize (Zea mays L.). The plastids undifferentiated (pun) mutation disrupts plastid biogenesis in both bundle sheath and mesophyll cells, at an early developmental stage and in a light-independent manner. Chloroplast thylakoids are disrupted in the mutant and both membrane-associated and soluble chloroplast-encoded proteins accumulate at much reduced levels. The observed plastid morphology is consistent with a general defect in chloroplast biogenesis that is most likely exerted at the post-translational level. Despite aberrant chloroplast development, nuclear photosynthetic genes are expressed normally in pun mutants. Thus, neither functional chloroplasts nor the Pun gene product are required to establish nuclear photosynthetic gene expression patterns in maize.

Keywords Cellular differentiation • Chloroplast biogenesis • C4 photosynthesis • Mutant maize (pun) • Zea (plastid differentiation)

Abbreviations LHCPII: chlorophyll a/b-binding protein of photosystem II • Lsu: large subunit of Rubisco • MDH: NADP-dependent malate dehydrogenase • ME: NADP-dependent malic enzyme • PEPCase: phosphoenolpyruvate carboxylase • PPDK: pyruvate phosphate dikinase • RuBPCase: ribulose bisphosphate carboxylase • SBPase: sedoheptulose bisphosphatase • Ssu: small subunit of Rubisco

Introduction

The development of photosynthetic competence in any plant relies on intracellular signalling mechanisms. Nuclear, chloroplast and mitochondrial genomes all contribute to photosynthetic differentiation but the way in which gene activity is co-ordinated is not entirely understood. Analysis of nuclear mutations that disrupt photosynthesis has shown that nuclear genes can regulate chloroplast gene expression (reviewed in Taylor 1989). Conversely, studies of carotenoid-deficient seedlings in which chloroplast development is disrupted (induced either by mutation or by norflurazon application) have suggested that there are factors synthesised in the chloroplast required for nuclear photosynthetic gene expression (reviewed in Oelmuller 1989; Taylor 1989). For example, photooxidative destruction of chloroplast function is correlated with decreased expression of the nuclear ribulose bisphosphate carboxylase (RuBPCase) small subunit (RbcS) genes and also of the nuclear Cab genes that encode the chlorophyll a/b-binding protein of photosystem II (LHCPII) (Batschauer et al. 1986; Mayfield and Taylor 1984, 1987; Oelmuller 1989).

In plants that utilise the C4 photosynthetic pathway, intercellular signalling mechanisms are also required during photosynthetic development because two photosynthetic cell types co-operate to fix carbon (reviewed in Edwards and Walker 1983). The most extensively studied C4 plant, maize, exhibits a classical Kranz-type anatomy where veins are surrounded by concentric files of photosynthetic bundle sheath and mesophyll cells
(Brown 1975). Mesophyll cells develop small chloroplasts with stacked grana that are arranged randomly within the cell and contain the majority of photosystem II activity present in the leaf (Meierhoff and Westhoff 1993; Wrischer 1989). The C₄ carbon-shuttle enzymes phosphoenolpyruvate carboxylase (PEPCase), NADP-dependent malate dehydrogenase (NADP-MDH) and pyruvate phosphate dikinase (PPDK) accumulate and act specifically in mesophyll cells. In contrast, the thick-walled bundle sheath cells develop agranal chloroplasts that accumulate the C₄ decarboxylating enzyme NADP-dependent malic enzyme (NADP-ME) and enzymes of the Calvin cycle. C₄ physiology thus requires the spatial restriction of Calvin cycle enzymes to the bundle sheath and the cell-specific accumulation of enzymes that catalyse the reactions involved in the intercellular carbon-shuttle. Although C₄ differentiation patterns have been characterised extensively (reviewed in Nelson and Langdale 1992) the signals required for specialisation are still unidentified.

To identify nuclear genes that are required for the development of photosynthetic competence in maize, we have screened transposon-containing lines for photosynthetically defective mutants. Work reported here describes a phenotypic characterisation of the plastids undifferentiated (pun) mutant. In mutant plants, plastid morphology is aberrant throughout development in both the light and the dark. Internal chloroplast membranes are not apparent and thylakoid-associated proteins do not accumulate. Despite perturbed chloroplast development, nuclear photosynthetic gene expression is essentially normal in light-grown pun leaves. The reported observations are consistent with a general defect in pun mutants that disrupts the accumulation of chloroplast-encoded proteins at the post-translational level.

Materials and methods

Plant material and growth conditions

The plastids undifferentiated (pun) allele was isolated from a maize (Zea mays L.) line containing active Spm transposable elements and was previously named bundle sheath defective3 (bsd3) (Langdale et al. 1995). pun plants are yellow, do not exhibit a variegated phenotype and die after the endosperm reserves are depleted (normally approx. 13 days after planting). Pun/pun heterozygous plants were crossed and backcrossed into the inbred line W22. Heterozygous progeny of the second cross into W22 were self-pollinated, and the resultant progeny were used for the experiments described in this report. In all cases, pun mutants segregated one in four, indicating the recessive nature of the mutation.

Seedlings were grown in soil in a growth chamber maintained at 28 °C with a 16 h light/8 h dark cycle. Plants were grown under moderate (100 μmol photons m⁻² s⁻¹) or low (10 μmol m⁻² s⁻¹) irradiance levels. Plastochron 1–5 (P1–5) leaf primordia were harvested 6 days after planting when seedlings were 1.5–2 cm tall and all leaves were still enclosed in the coleoptile. For all experiments, the young shoots were excised 2–3 mm above the shoot apical meristem, after which the seedlings were transferred to the growth chamber and grown until mutant phenotypes could be scored. Two 2- to 3-mm transverse sections were taken from the base of the immature shoot for in situ experiments, thus comprising P3–P5 leaf primordia enclosed within an outer coleoptile. For transmission electron microscopy (TEM), the coleoptile and all internal leaf primordia were removed before harvesting 2-mm² tissue samples across the base of leaf 1 (plastochron 5). Three leaves were harvested 11 days after sowing, as the fourth leaf was emerging. For in situ and TEM analyses, tissue samples were taken 1 cm from the base and 1 cm from the tip of the leaf.

Etiolated seedlings were grown in vermiculite in complete darkness at 28 °C for 6 days until the first 1 cm of the unexpanded first leaf was visible above the coleoptile. Tissue was harvested by excising the seedling 1 cm above the shoot apical meristem (under a green safelight), after which plants were transferred to the growth chamber and grown until mutant phenotypes could be scored. For RNA and immunoblot analyses, the coleoptile was removed prior to harvesting. Tissue samples for TEM analysis were taken within 1 cm of the tip of leaf 1. Light-shifted seedlings were grown in complete darkness, as described above, and then shifted to the growth chamber for 24 h. Seedlings were shifted to either moderate (100 μmol m⁻² s⁻¹) or low (10 μmol m⁻² s⁻¹) light intensities.

Measurement of photosynthetic pigments

Photosynthetic pigments in light-grown leaves were measured as described in Lichtenthaler and Wellburn (1983). Briefly, pigments were extracted in 80% acetone (v/v) at 4 °C and the slurry cleared by centrifugation at 10,000 g for 5 min. The absorbance was read in a SP8-100 UV/VIS PYE UNICAM Spectrophotometer. Pigment concentrations were estimated according to the absorbance coefficients calculated by Lichtenthaler and Wellburn (1983) and expressed as μg of pigment per cm² of leaf area.

Antibody and cDNA probes

Antiseras raised against Rubisco holoenzyme (Triticum aestivum), Rubisco large subunit (Lsu) (Flaveria bidentis), Rubisco small subunit (Ssu) (maize), PEPCase (Amaranthus hypochondriacus), PPDK (maize), NADP-ME (maize), and NADP-MDH (maize) have been described previously (Langdale et al. 1987; Langdale and Kidner 1994). Sedoheptulose bisphosphatase (SBPase) antibody (wheat) was a gift from T. Dyer (John Innes Institute, Norwich, UK). Vacuolar ATPase antibody (Kalanchoe) was a gift from J.A.C. Smith (University of Oxford). eIF4A antibody (tobacco) was a gift from C. Kuhlemeier (University of Bern, Switzerland). With the exception of Lsu and eIF4A antisera, all antibodies used were polyclonal.

Ribulose bisphosphate carboxylase (RuBPCase) small subunit (Rbs-S), RuBPCase large subunit (Rbs-L), PEPCase (Ppc1), NADP-ME (Modl), NADP-MDH (Mdh1), and PPDK (Ppdk1) cDNA clones (pJL10, pJL12, pTN1, pTN5, C30, and pH2, respectively) were as described in Roth et al. (1996). LHCP1I (Cab) cDNA clone pZLH5 was a gift from Bill Taylor (CSIRO, Canberra, Australia). Ubiquitin cDNA (pSkuB1) was a gift from Peter Quail (Plant Gene Expression Center, Albany, Calif., USA).

Preparation of total leaf protein and immunoblot analysis

The isolation of total leaf protein and immunoblot analysis was carried out as in Roth et al. (1996).

Preparation of RNA and RNA gel blot analysis

RNA was purified, electrophoresed and blotted as reported previously (Roth et al. 1996).

Preparation of polysomes

A modification of the procedure of Klaff and Gruissem (1991) was used to isolate total polysomes from leaf tissue. Light-grown
seedling tissue (0.5 g) was ground in liquid nitrogen to a fine powder and added to 1.5 ml buffer. Homogenate was purified and adjusted to 0.5% sodium-deoxycholate as described previously (Klaff and Gruissem 1991). Aliquots of 0.5 ml were layered onto 10.5 ml 15% to 45% sucrose gradients in 40 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl₂, 0.5 mg/ml heparin, 100 μg/ml chloramphenicol and centrifuged for 150 min at 40,000 rpm in a Sorvall (DuPont, Wilmington, Del., USA) Ti 41 rotor at 4 °C. Ten fractions of 650 μl were collected corresponding to the central region of the gradient. Fractions were adjusted to 0.5% SDS, 20 mM EDTA and 80 mM Tris-HCl (pH 9.0). RNA was extracted with 1:1 (v/v) phenol:chloroform and then 24:1 (v/v) chloroform:isoamyl alcohol prior to precipitation with 2-propanol. RNA was fractionated on agarose gels and hybridised with a fragment of maize chloroplast DNA (pZMC 460) containing both rbcL and atpB sequences (kindly provided by A. Barkan, University of Oregon, USA).

In situ localisation of C4 gene products

Plant tissues were fixed in 3:1 (v/v) ethanol/acetic acid for 30 min at room temperature and stored at 4 °C in 70% ethanol. Samples were embedded in Paraplast Plus (Sherwood Medical Co., St Louis, Mo., USA) and sectioned to 8 μm as reported previously (Langdale et al. 1987). RNA in situ hybridisation assays, were carried out using 32P-labelled riboprobes as described in Langdale and Kidner (1994).

Electron microscopy

All tissues were cut under and fixed in Karnovsky’s fixative (3% paraformaldehyde; 3% glutaraldehyde; 0.025 M phosphate buffer, pH 7.2) for 2 h at room temperature. All plant tissues that needed to be scored for a mutant phenotype, e.g. etiolated and P1–5 leaves, were stored in 0.025 M phosphate buffer (pH 7.2) after pre-fixing (for a maximum of 7 days). Post-fixation treatments, embedding in TAAB resin (TAAB Laboratory Equipment, Reading, UK), sectioning and staining were carried out as previously reported (Roth et al. 1996). Sections were examined on a JEOL JEM-2000EX transmission electron microscope and photographed using AGFA Scientia EM film 23 D 56 (Leverkusen, Germany).

Results

The pun mutation disrupts chloroplast biogenesis in both bundle sheath and mesophyll cells

Segregation analyses indicated that the pun mutant phenotype is caused by a single recessive nuclear mutation. Leaves of pun plants are yellow and do not green at any stage of development unless plants are grown in very dim light (data not shown). Mutant plants die once the endosperm reserves have been depleted. To investigate the extent to which the pun mutation disrupts pigment biosynthesis, dark-grown seedlings were shifted to low (10 μmol m⁻² s⁻¹) or moderate (100 μmol m⁻² s⁻¹) light for 24 h. Unlike wild-type (Pun) siblings, which greened rapidly after exposure to light, pun leaves remained yellow and failed to accumulate any visibly detectable chlorophyll (data not shown). This suggested that the Pun gene might have a role in synthesis or maintaining normal chlorophyll levels. To quantify this further, pigment concentrations were measured in low- and moderate-light-shifted leaves. As shown in Table 1, when pun mutant leaves were exposed to low light conditions, chlorophyll a, chlorophyll b and carotenoids accumulated to 24%, 29% and 48% of wild-type levels, respectively. Under moderate light conditions, chlorophylls a and b were reduced by 94% and 92%, respectively, while carotenoid levels were reduced by 65% compared to the wild type. These data suggest that any chlorophyll that is synthesised in mutant leaves is rapidly degraded under moderate light. We cannot exclude the possibility that the Pun gene is directly involved in pigment biosynthesis and that the low levels of chlorophyll and carotenoids observed are due to incomplete penetrance of the pun mutation. However, the pigment levels seen in plants grown in low light suggest that there is not a major biosynthetic block in either pathway.

To investigate the extent to which chloroplast development is disrupted in pun mutant seedlings, chloroplast ultrastructure was examined in young leaf primordia and in seedling leaves grown in moderate light (100 μmol m⁻² s⁻¹). At plastochron 5 (P5), bundle sheath and mesophyll progenitor cells were cytoplasmically dense and could be distinguished only by their position relative to vein sites; plastids in both cell types were identical (Fig. 1A). (A plastochron is the time interval between the initiation of successive leaf primordia.) Figure 1B shows that in pun mutant P5 leaf primordia, plastid structure was aberrant in both bundle sheath and mesophyll progenitor cells. Plastids were reduced in size, were highly vesiculated and lacked the primary thylakoid lamellae characteristic of wild-type plastids at this early developmental stage. Interestingly, electron-dense particles that resemble osmiophilic

<table>
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<th>Material</th>
<th>Chl a (μg cm⁻²)</th>
<th>Chl b (μg cm⁻²)</th>
<th>Chl a/b</th>
<th>% Normal Chl a</th>
<th>% Normal Chl b</th>
<th>Carotenoids (μg cm⁻²)</th>
<th>% Normal</th>
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<td>Pun</td>
<td>17.31 (6.55)</td>
<td>2.85 (0.97)</td>
<td>6.07</td>
<td>100</td>
<td>100</td>
<td>5.22 (1.72)</td>
<td>100</td>
</tr>
<tr>
<td>pun</td>
<td>1.05 (0.54)</td>
<td>0.24 (0.83)</td>
<td>4.45</td>
<td>6</td>
<td>8</td>
<td>1.83 (0.14)</td>
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<tr>
<td>Pun</td>
<td>11.96 (1.66)</td>
<td>1.93 (0.48)</td>
<td>6.20</td>
<td>100</td>
<td>100</td>
<td>3.89 (0.46)</td>
<td>100</td>
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<tr>
<td>pun</td>
<td>2.90 (1.34)</td>
<td>0.56 (0.23)</td>
<td>5.16</td>
<td>24</td>
<td>29</td>
<td>1.88 (0.13)</td>
<td>48</td>
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particles were present along the plasma membrane of both cell types in *pun* mutant leaf primordia. Photooxidative damage is unlikely to occur at this stage in development since leaves are enclosed within the coleoptile and are therefore not exposed to a substantial amount of light. Thus, the chloroplast morphology observed at P5 most likely reflects a primary effect of the *pun* mutation.

As a consequence of normal maturation patterns, the strap-shaped maize leaf presents a developmental gradient with immature cells at the base and the most mature cells at the tip of the leaf blade (Sharman 1942; Kirchanski 1975; Sylvester et al. 1990). To determine the effect of the *pun* mutation on plastid biogenesis, chloroplast ultrastructure was examined in leaf sections taken at the base and tip of wild-type (Fig. 1C, E) and mutant (Fig. 1D, F) third leaf blades grown in moderate light (100 μmol m⁻² s⁻¹). During all stages of leaf development, chloroplast structure in both bundle sheath and mesophyll cells was aberrant. At the base of the *pun* mutant leaf, chloroplasts were vesiculated and lacked the internal lamellae present in the wild type (Fig. 1D). Vesiculation was more pronounced at the leaf tip and was observed in both bundle sheath and mesophyll cell chloroplasts (Fig. 1F). Occasionally, mesophyll cells were observed with chloroplasts that exhibited rudimentary thylakoid stacking (Fig. 1G, H). The existence of these more-normal-looking mesophyll chloroplasts at the leaf tip suggests either that the *pun* mutation is a partial-loss-of-function allele, such that some PUN protein accumulates in older cells, or that a second gene can compensate for loss of PUN activity. In mature cells at the tip of the leaf, it is possible that some aspects of the chloroplast phenotype are due to photooxidative damage and hence represent secondary effects of the mutation. However, aberrant plastid morphologies seen at the base of mutant leaves demonstrate that the wild-type *Pun* gene is essential for normal chloroplast biogenesis.

To distinguish between primary and secondary effects of the *pun* mutation on chloroplast ultrastructure, we examined ultrathin sections of etiolated and light-shifted leaves. In etiolated wild-type leaves, both the bundle sheath and mesophyll cell plastids contained a crystalline prolamellar body as shown in Fig. 2A. In *pun* mutant leaves, however, etioplasts appeared highly vesiculated, lacked primary thylakoid lamellae and contained only rudimentary prolamellar bodies (Fig. 2B). The failure of *pun* mutant plastids to differentiate normally in the dark suggests that PUN plays a light-independent role in plastid biogenesis. Upon exposure to light, wild-type etioplasts differentiate rapidly into chloroplasts (Fig. 2C; Bradbeer 1981). However, after *pun* mutant seedlings were exposed to moderate light (100 μmol m⁻² s⁻¹) for 24 h, etioplasts did not differentiate into chloroplasts insofar as rudimentary prolamellar bodies could still be observed (Fig. 2D). To determine whether the observed defect in etioplast-to-chloroplast conversion seen in *pun* mutant seedlings was due to photooxidative damage, chloroplast ultrastructure was also examined in seedlings shifted to low light (10 μmol m⁻² s⁻¹) (Fig. 2E, F). No differences in chloroplast morphology could be observed between seedlings that were shifted to moderate versus low light. Thus, aberrant chloroplast structure in light-shifted *pun* mutant leaves is primarily due to developmental defects.

Thylakoid-associated proteins accumulate to reduced levels in *pun* mutants

Electron micrographs suggested that internal membranes in *pun* mutant chloroplasts are severely disrupted (Figs. 1, 2). To characterise this disruption further, we compared accumulation levels of thylakoid-associated proteins in wild-type and mutant seedlings. Leaf membrane proteins were extracted from etiolated seedlings, seedlings shifted to low light for 24 h (10 μmol m⁻² s⁻¹) and seedlings shifted to moderate light for 24 h (100 μmol m⁻² s⁻¹). That is, proteins were isolated from leaves exhibiting the plastid phenotypes shown in Fig. 2. Immunoblots were reacted with antibodies raised against proteins that represent three of the major photosynthetic complexes (PSII, ATPase and Cyt f/b6). Figure 3A shows that nuclear-encoded LHCP II accumulates to lower levels in *pun* mutant plants than in wild-type siblings, while the chloroplast-encoded proteins CF1a and Cyt f are undetectable in mutant tissue. Subunits of the membrane-localised vacuolar ATPase were detected in mutant samples at levels at least equivalent to those in the wild type (Fig. 3B). Thus, there is a specific decrease in the level of chloroplast membrane proteins in *pun* mutants.

The accumulation profile of LHCP II reveals three important points about the *pun* mutation. Firstly, since decreased levels of LHCP II are most apparent in plants shifted to moderate light intensity, some of the perturbations seen in mutant chloroplasts may be due to photooxidative damage of the thylakoid membranes. Secondly, as LHCP II protein is undetectable in dark-grown mutant plants but accumulates within 24 h of exposure to low light, light-signalling pathways are intact in *pun* mutant plants. Thirdly, since nuclear-encoded LHCP II accumulates within *pun* plastids (albeit at reduced levels), chloroplast import pathways are at least partially functional in the mutant.

Our failure to detect CF1a or Cyt f protein in mutant leaves (Fig. 3A) suggests that transcription and/or translation of the chloroplast genes *atpA* and *petA* is perturbed in *pun* mutants. Furthermore, since the absence of individual subunits often indicates coordinate loss of the entire complex (Barkan et al. 1986), it can be assumed that the Cyt f/b6 complex and the chloroplast ATPase fail
Fig. 2 Transmission electron microscopy of etiolated and light-shifted leaves of wild-type (A, C, E) and pun mutant (B, D, F) seedlings of maize. A, B Etiolated leaves; C, D greening leaves after a 24-h shift to moderate light (100 μmol m$^{-2}$ s$^{-1}$); E, F greening leaves after a 24-h shift to low light (10 μmol m$^{-2}$ s$^{-1}$). BS bundle sheath, $M$ mesophyll, $rpb$ rudimentary prolamellar body. Bar = 1 μm.

Rubisco Lsu protein does not accumulate in pun mutant leaves.

The observations described above suggest that proteins encoded by the chloroplast genome do not accumulate...
in *pun* mutant plants. To determine whether this effect is limited to membrane-associated proteins or instead represents a more general defect in chloroplast gene expression mechanisms, we assayed the accumulation pattern of the stromal protein Rubisco Lsu in the wild type and in *pun* mutant seedlings. Plants were grown in low light (10 μmol m⁻² s⁻¹) to avoid possible secondary perturbations due to photooxidative damage. Figure 4A shows that Lsu protein was virtually undetectable in *pun* mutant leaves. This observation supports the idea that the *pun* mutation may condition a general defect that perturbs the accumulation of all proteins encoded by the chloroplast genome.

To determine the level at which *rbcL* gene expression is perturbed in *pun* mutants, we investigated the extent to which *rbcL* transcripts accumulated and are translated. Northern blot analysis showed that *rbcL* transcripts accumulated to approx. 50% of normal levels (as determined by densitometry) in *pun* mutant leaves grown in low light (10 μmol m⁻² s⁻¹) (Fig. 4B). Thus, *rbcL* transcription or transcript stabilisation processes are at least partially defective in *pun* mutants. Relative to the wildtype, however, Lsu protein levels were decreased to a greater extent than *rbcL* transcript levels. To investigate this further, we examined the association of *rbcL* transcripts with polysomes. The extent to which transcripts are associated with polysomes, provides a means to examine the efficiency of translation initiation and elongation (Barkan 1993; Berry et al.1988). RNA was extracted from wild-type and *pun* mutant tissue, fractionated on 15% to 45% sucrose gradients to separate polysome-associated transcripts from monosomes and free RNA, and then hybridised to *atpB* and *rbcL*. As shown in Fig. 4C, *atpB* and *rbcL* transcripts from wild-type and mutant plants sedimented at similar rates. Furthermore, the primary 1.8-kb *rbcL* transcript was processed correctly into a 1.6-kb transcript and both transcripts associated with polysomes in *pun* mutants. Since translation initiation and the early steps of elongation proceed normally in *pun* mutants, the failure to accumulate Lsu protein must therefore be due to a post-translational defect.

Nuclear photosynthetic gene expression is essentially normal in light-grown *pun* mutant leaves

Previous studies of carotenoid-deficient seedlings suggested that plastid integrity is required for the production of plastid factor(s) which induce transcription of the nuclear genes *Cab* and *RbcS* (reviewed in Oelmuller 1989). We have established that plastid integrity is severely perturbed in *pun* mutants. A number of chloroplast-encoded proteins fail to accumulate (Figs. 3, 4), chloroplast gene expression is perturbed at more than one level (Fig. 4) and chloroplast morphology is aberrant throughout development (Figs. 1, 2). To examine the extent to which these plastid aberrations influence
nuclear gene expression, *Cab* and *RbcS* transcript levels were compared in wild-type and *pun* mutant seedlings that were either grown in total darkness, in low light (10 μmol m⁻² s⁻¹) or in moderate light (100 μmol m⁻² s⁻¹). Figure 5A shows that *Cab* and *RbcS* transcript levels were slightly reduced in light-grown *pun* mutant plants, most noticeably in plants grown in moderate light. This observation suggests that if plastid factors are required for *Cab* and *RbcS* gene expression, those factors are at least partially functional in *pun* mutants and that the decreased transcript levels in plants grown in moderate light may result from photooxidative damage. Notably, however, *RbcL* transcript levels were also reduced in etiolated *pun* plants where photooxidative damage cannot occur. *rbcL* transcript levels were similarly perturbed in both dark- and light-grown plants (Fig. 5B), supporting the idea that

PUN plays a light-independent role in chloroplast biogenesis.

To determine the effect of aberrant plastidogenesis on other nuclear photosynthetic genes, we analysed accumulation patterns of transcripts encoding the C₄ carbon-shuttle enzymes. Figure 5C shows that levels of transcripts encoding PEPCase, PPDK, ME and MDH were normal in etiolated, low-light-grown and moderate-light-grown *pun* mutant tissue. Notably, even growth at moderate light, where some degree of photooxidative damage may occur (see above) did not lead to a decrease in transcript levels. Thus, aberrant plastid biogenesis conditioned by the *pun* mutation does not influence C₄ gene expression patterns.

C₄ carbon-shuttle and Calvin cycle enzymes accumulate in *pun* mutants

To determine whether nuclear-encoded photosynthetic proteins are synthesised and accumulate in *pun* mutants, leaf proteins were extracted from seedlings that were grown in the dark and then shifted to low light (10 μmol m⁻² s⁻¹) or moderate light (100 μmol m⁻² s⁻¹). Immuno- blots were reacted with antibodies raised against cytosolically localised (PEPCase) and plastid-targeted (NADP-ME, PPDK, NADP-MDH) carbon- shuttle enzymes and against representative Calvin cycle enzymes [sedoheptulose bisphosphatase (SBPase) and Rubisco Ssu and Lsu]. Figure 6A shows that all of the carbon-shuttle enzymes accumulated to essentially normal levels in *pun* mutant leaves. Similarly, SBPase accumulated to normal levels (Fig. 6B), suggesting that there is no general decrease in Calvin cycle proteins. The only nuclear-encoded photosynthetic protein that failed to accumulate to normal levels in *pun* mutant leaves was Rubisco Ssu. However, since *RbcS* transcripts accumulated (Fig. 5), it is likely that Ssu protein levels are compromised due to aberrant accumulation of the Lsu protein (Rodermel et al. 1988; Avni et al. 1989; Jiang and Rodermel 1995) rather than as a direct result of either the *pun* mutation or aberrant plastid biogenesis.

Spatial and temporal patterns of *RbcS* and *rbcL* transcript accumulation are normal in *pun* mutants

To determine whether reduced Rubisco levels in *pun* mutants are coupled to aberrant spatial and/or temporal patterns of gene expression, *rbcL* and *RbcS* transcripts were examined in situ in wild-type and mutant PI–5 leaf primordia and seedling leaves grown in moderate light (100 μmol m⁻² s⁻¹), i.e. in tissue exhibiting the plastid phenotypes shown in Fig. 1. In wild-type leaves, *rbcL* mRNAs accumulate specifically in bundle sheath progenitor cells between P4 (surrounding major vein sites) and P5 (surrounding intermediate vein sites) (Langdale et al. 1988a). As shown in Fig. 7A, although the levels of *rbcL* transcripts were reduced, the timing of
Fig. 6A—C Immunoblot analysis of soluble proteins extracted from wild-type (Pun) and mutant (pun) maize seedlings. Seedlings were grown in the dark and then shifted for 24 h to low (10 μmol m⁻² s⁻¹) or moderate (100 μmol m⁻² s⁻¹) light. A Immunoreaction with antibodies raised against the carbon-shuttle enzymes PEPCase, PPDK and NADP-ME, NADP-MDH. B Immunoreaction with antibodies raised against the Calvin cycle proteins SBPase and Rubisco (Lsu and Sau). C Loading control: immunoreaction with antibody raised against the translation factor eIF4A. Protein was isolated using a standard weight of tissue per volume of extraction buffer, using the same tissue as used for the insoluble proteins shown in Fig. 3. Equal sample volumes were loaded in each lane. Respective molecular masses (kDa) are indicated on the left.

rbcL transcript accumulation in pun mutant leaf primordia appeared to be the same as in the wild-type. Furthermore, in both leaf primordia and third leaf blades, rbcL transcripts were localised specifically in bundle sheath cells (Fig. 7A). Similarly, spatial and temporal patterns of Rubisco transcript accumulation were unperturbed (Fig. 7B). These results suggest that the spatial regulation of Rubisco gene expression does not depend on the presence of fully functional chloroplasts or on a functional Pun gene product.

Discussion

Mutations that disrupt pigment accumulation are very common in higher plants owing to the large number of nuclear- and chloroplast-encoded gene products that are essential for photosynthesis. Pigment-deficient mutants in maize have therefore been divided into two groups to distinguish those with perturbed pigment biosynthetic pathways from those with developmental defects (Coe et al. 1988). Class-I mutants, such as lemon white and white3 are blocked in the carotenoid biosynthetic pathway. These mutants are partially or fully pigmented in dim light but are completely bleached by moderate light as a result of photooxidative damage (Robertson 1975). Similarly, chloroplast morphology is more severely disrupted in moderate-light-grown as compared to dim-light-grown plants (reviewed in Robertson 1975). In contrast, yellow and pale-green class II mutants represent a group of mutations that disrupt chloroplast biogenesis or chloroplast gene expression in a light-independent manner (Han et al. 1993, 1995). Although some photooxidative damage is observed in pun mutants, particularly with respect to pigment concentrations and LHCPII accumulation, plastid morphology is perturbed in a light-independent manner. Based on these observations, the pun mutant falls within class II and is therefore unlikely to be defective in the carotenoid biosynthetic pathway. We also believe that the Pun gene does not have a direct role in the chlorophyll biosynthetic pathway, primarily because chloroplast structure in pun mutants is disrupted as early as P5 yet chlorophyll biosynthetic mutations characteristically cause chloroplast biogenesis to be arrested during relatively late stages of chloroplast development (Mascia and Robertson 1978; Hudson et al. 1993; Cowan 1995; Runge et al. 1995).

The morphological features of the pun mutant phenotype suggest that the Pun gene product facilitates normal chloroplast biogenesis in both the light and the dark. In the absence of PUN, chloroplast biogenesis is perturbed very early in development (at least as early as P5). Chloroplasts fail to develop normal membrane structures and there is a concomitant decrease in accumulation of thylakoid-associated photosynthetic complexes. Notably, chloroplast-encoded thylakoid proteins are more severely affected than the nuclear-encoded LHCPII protein. This observation suggests that the pun mutation may condition a general defect that affects the accumulation of chloroplast-encoded proteins. In support of this idea, chloroplast-encoded rbcL transcript and protein levels are also reduced in mutant plants. Since Lsu protein levels are more severely perturbed than rbcL transcript levels and rbcL transcripts load onto polysomes, the pun mutation probably does not condition a general defect in the chloroplast transcriptional machinery or in translation initiation events. It is therefore likely that PUN plays either a specific or widespread role in the post-translational regulation of chloroplast-localised complexes. A number of observations support the suggestion that PUN has a general role in these post-translational processes. For example, characterisation of the maize mutant bundle sheath defective 2 (bsd2) has shown that specific loss of Rubisco protein is not accompanied by developmental defects in plastid biogenesis programs (Roth et al. 1996; Brutnell et al. 1999). Similarly, characterisation of the maize psa, psb and pet mutants has shown that individual chloroplast membrane complexes can be perturbed without affecting other chloroplast components (Barkan et al.
Fig. 7 In situ detection of rbcL (A) and RbcS (B) transcripts in plastochron 4/5 (P4/5) leaf primordia and third leaf blades of wild-type (Pun) and mutant (pun) maize siblings grown in moderate light (100 μmol m⁻² s⁻¹). Bar = 50 μm

Previous work has suggested that plastid signals are required for the accumulation of nuclear-encoded RbcS and Cab transcripts (reviewed in Oelmuller 1989; Taylor 1989). In pun and other maize yellow mutants (Mayfield and Taylor 1984), RbcS and Cab transcript levels are essentially normal. Thus, plastid signals are intact in these mutants despite aberrant plastid morphology. The accumulation of both carbon-shuttle and Calvin cycle
enzymes in *pun* mutants suggests that neither the *Pun* gene product nor chloroplast function is required to establish C₄ photosynthetic gene expression patterns. It has previously been proposed that during photomorphogenesis in maize, light-enhanced signals emanate from the leaf veins to pattern bundle sheath and mesophyll cells (Langdale et al. 1988b). Cells perceive and respond to these signals on the basis of their distance from the vein. Since spatial and temporal patterns of gene expression are unperturbed in *pun* mutants, bundle sheath and mesophyll cells adopt their appropriate fate. Thus, the proposed light-enhanced positional signals that emanate from the vein to establish the spatial patterning of photosynthetic gene expression are not dependent on chloroplast development or function.

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